A SIMPLE METHOD FOR PRESERVING BACTERIAL CULTURES BY FREEZING AND DRYING[1]

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Numerous studies on colony forms and on antigenic structure and virulence of bacteria have demonstrated conclusively that these microörganisms are constantly undergoing significant variation when they are repeatedly subcultured on artificial media or subjected to animal passage. The desirability of maintaining type-culture collections, as well as cultures for use in any laboratory, in the state in which the bacteria were originally isolated is obvious; while the cost of artificial media and the labor involved in frequent subculturing are additional incentives for establishing suitable techniques for satisfactorily preserving bacteria. Drying while in the frozen state has been shown to accomplish this end; the viability, virulence, immunological and biochemical characteristics of bacteria and certain other infectious agents are all maintained. For example: we have recently found, viable and still possessing their group specific substance, cultures of hemolytic streptococci preserved in this way in 1916–17. Many other strains of hemolytic streptococci have been similarly stored in this laboratory within the past twelve years during the course of studies of their antigenic components and colony forms (Lancefield, 1933); these have been recovered at intervals and have consistently maintained the characteristics noted at the time of their original preservation.[2] Pneumococci similarly preserved have kept their type specificity. Rake has found that certain strains of pneumococci, having a very labile

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[2] I am indebted to Dr. R. C. Lancefield for these studies.

capacity to invade the blood stream of mice through the nasal mucosa, quickly lose this capacity unless they are preserved by freezing and drying. He (1935) has further observed that such delicate bacteria as meningococci have maintained their original virulence and specific carbohydrate content for at least 20 months when preserved in the same manner.

Elser, Thomas and Steffen (1935) have had similar experiences with many strains of bacteria. Filterable viruses may also be preserved if infected tissues or exudates are frozen and then dried; for instance, the yellow fever virus has been kept for at least five years in the Laboratories of the International Health Board (Sawyer and Bauer).

In most of the work mentioned above the bacteria were frozen in a salt-ice mixture; but there is reason to believe that protoplasm may be less injured when more quickly frozen at −76°C. Turner (1936) has recently maintained the viability and virulence of *Treponema pallidum* at this low temperature for 4 to 6 months, while, on the other hand, several years ago I was unable to demonstrate any viability in similar treponemas in rabbit chancres frozen at −6° to −10°C. In the frozen food industry it has been shown that much smaller crystals are formed in fish quickly frozen at −76°C. than in that more slowly frozen at higher temperatures (Fitzgerald), and it seems probable that the smaller crystals are less harmful to the frozen cell.

Many bacteria will live in a simple dried state for considerable lengths of time, with gradual reduction in viability, a fact that has led to the method of preserving cultures by drying them, without freezing, on filter paper or other fibrous or granular material. When bacteria are suspended in a colloid, such as serum or blood, and then dried without preliminary freezing, they may retain their original characteristics for many months. Recently Stillman has observed this phenomenon with pneumococci dried in defibrinated rabbit blood, where control cultures dried simply in broth succumbed in a few weeks. Dried infected exudates and tissues, such as spleens, are also useful agents for preserving some bacteria.

A combination of freezing and drying for the preservation of biological products was suggested years ago by Shackell (1909)

and used shortly afterwards for keeping rabies virus (Harris and Shackell, 1911), and bacteria (Hammer, 1911). We improved the technique a few years later and described the method and results of its application in 1921 (Swift). Elser has accomplished the same purpose with a different form of apparatus which he and his collaborators have recently described (Elser, Thomas and Steffen, 1935). Their article also contains a good review of the literature on this subject. Later, Sawyer and his co-workers (Sawyer, Lloyd and Kitchen, 1929) devised an apparatus which is automatic in its action; and still more recently Flosdorf and Mudd (1935) have perfected their so-called "lyophile" apparatus which has many excellent points.

The principle involved in all techniques is the removal of the water from the material while it is still frozen; thus a harmful concentration of salts and other substances is obviated. Two methods are employed for removing the water from the frozen substances, both of which are carried out *in vacuo*. In one the water vapor is absorbed by a chemical desiccant; in the other it is removed by low temperature evaporation and condensation—a modified distillation. The latter is used in the apparatus of Elser, Thomas and Steffen and in that of Flosdorf and Mudd. These methods are specially applicable in the desiccation of immune sera and other biological products, since large quantities of water can be readily removed, and they also have the additional advantage that the special containers may be sealed with the contents under a high vacuum. The apparatus is, however, more expensive to purchase and to use than the more simple equipment here described, in which a chemical desiccant is employed. The apparatus described by Sawyer and his co-workers also makes use of a chemical desiccant. Its chief advantage is the certainty that the material will remain frozen until dried; but it is rather cumbersome and too expensive for many laboratories.

APPARATUS

The method originally described by us (Swift, 1921) and the modifications more recently developed can be carried out with equipment usually available. All methods require a good vacuum pump such as the Hyvac.

Desiccator. A desiccator with *well ground joints* and a *good stop cock* is essential. It contains glycerol, a manometer and a dish of P_2O_5. The ground glass surfaces are coated with a mixture, one part of paraffin, melting point 56°C., and 6 parts of vaseline. The small U-shaped mercury manometer is fastened to the inner side of the desiccator with adhesive plaster. Glycerol, with all traces of water removed, is placed in the bottom of the desiccator

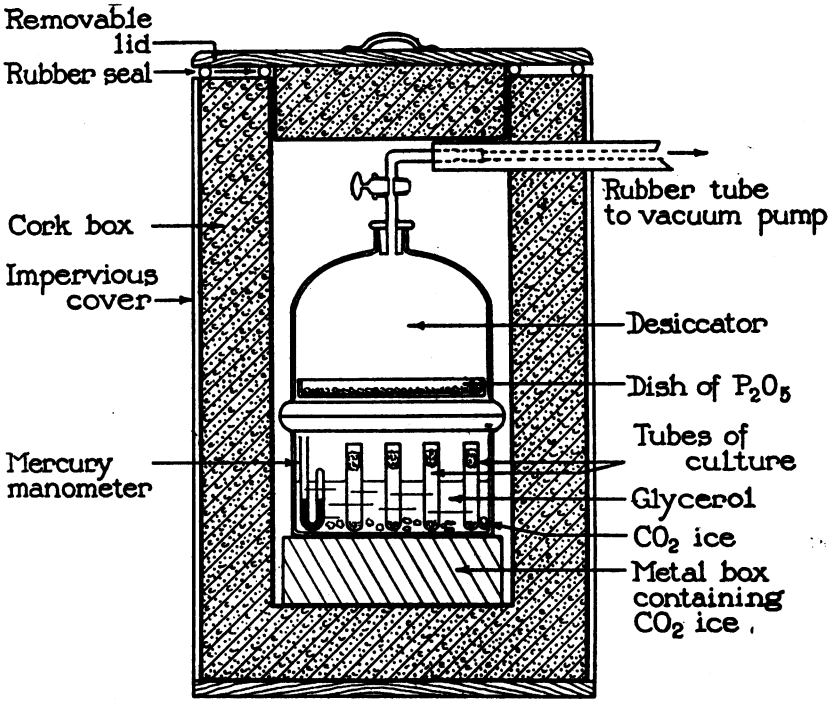

FIG. 1. DIAGRAM OF CORK REFRIGERATING BOX CONTAINING DESICCATOR EMPLOYED FOR FREEZING CULTURES WITH CO_2 ICE, AND DRYING WHILE IN THE FROZEN STATE

to a depth of 2 to 3 cm., and this glycerol is kept free of water by maintaining a vacuum in the desiccator when it is not in use. A large flat dish, such as half of a large Petri dish, is held in readiness for the desiccant P_2O_5, which is used as described below. This desiccant has proven more satisfactory than sulfuric acid or calcium chloride because of its more rapid action, as well as its greater hygroscopic power.

Refrigerating box. Since CO_2 ice has become readily available a simplified method of preserving cultures—called Method B—has been devised. For this a simple refrigerating box, that can be easily moved, has many advantages. Ours is built of 3-inch cork board which is glued together with a special cement.[3] The inside diameter is slightly larger than the desiccator, and the depth is sufficient to hold the latter and a metal box as shown in figure 1. The cork-board, insulating the removable top, is glued to a pine lid, and the bottom is also reinforced with a pine board. The sides of the box are entirely covered with closely woven canvas which is painted with several coats of impervious paint. The seal under the lid consists of two rows of rubber tubing. The lid is held in place simply by gravity, for a tight mechanical seal must be avoided on account of the pressure of CO_2 gas developed. A hole in the upper part of the refrigerating box permits the passage of heavy rubber pressure tubing from the pump to the desiccator. In the bottom of the cork box is placed a flat-topped sheet metal box containing CO_2 ice. The desiccator rests on this box.

TECHNIQUE OF METHOD B[4]

The refrigerator box is cooled by placing, from 200 to 400 grams of solid CO_2, in the metal container. The exact amount must be learned by experience with the conditions in each laboratory, for only enough is needed to keep the inside of the apparatus slightly below 0°C. until desiccation is complete. An excess will maintain a temperature so low that the process of drying is retarded.

Young, actively growing, broth cultures of the bacteria are centrifuged, the supernatant fluid is discarded, and the sediment is resuspended in broth or serum to about 1/25th of the original volume of culture.[5] In the case of bacteria which must be grown

[3] I wish to thank the Armstrong Cork Company for furnishing the cork board and cement and for useful suggestions in making the box.

[4] I am specially indebted to our technician, Stephen Nalesnyk, for many useful suggestions in the development of Method B.

[5] While in all of our work broth has been employed as a suspending medium, it seems that serum may be better in the case of extremely delicate bacteria or viruses. This is suggested by the experience of Stillman with the simple drying

on solid media, the surface growth is scraped off and suspended in broth or serum. The bacterial suspensions are distributed in small tubes (7 x 120 mm.) in 0.1 cc. amounts; and the cotton plugs are pushed down until their upper surface is about 5 mm. below the tops of the tubes. CO_2 ice is crushed and scattered over the top of the glycerol in the desiccator; the amount, varying between 300 and 500 grams, depends upon the quantity of material to be frozen. This CO_2 ice is quickly pushed to the bottom of the glycerol with the tubes of concentrated culture. The glycerol soon solidifies. The simultaneous introduction of the CO_2 and tubes results in the cultures being rapidly reduced to a temperature of about $-76°C$. The top is firmly pressed on the lower part of the desiccator so that a tight seal is formed between the ground glass surfaces which have been previously covered with a fresh coating of the paraffin and vaseline mixture. Sealing is facilitated by having the desiccator connected with the running vacuum pump; in fact, it is necessary to maintain a partial vacuum, because the rapid evolution of gas from the CO_2 ice may blow the top off the desiccator unless the gas is rapidly removed. The desiccator is placed in the cooled refrigerating box with as little interruption of the pumping as possible, and after an inspection of all connections the lid is placed on the cork box. Obviously, while the CO_2 is evaporating only a partial vacuum is possible, but during this period some water is probably being removed from the frozen cultures. The high vacuum required for satisfactory desiccation will not be attained until some time after the CO_2 is entirely evaporated; hence it is well to keep the pump running until an hour or more after this evaporation is completed. For this reason it is advisable not to put too much CO_2 ice in the desiccator, only enough to insure rapid freezing of the cultures, and maintenance of the frozen state until the cultures are dry. The amounts will depend upon the size of the

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of pneumococci. Furthermore, Rivers and Ward (1935) have noted that cultures of vaccine virus remain viable much longer in the dried state following freezing when they are previously mixed with a colloid such as 3 per cent gum acacia; and Dochez and his coworkers (Dochez, Mills, and Kneeland, 1936) have observed the same with cultures of virus of the common cold.

apparatus, the initial temperature of the glycerol, and the amount of material to be treated. It is convenient to have a good vacuum gauge, connected by means of a three-way stop cock situated between the desiccator and the pump, in order to determine when a sufficiently high vacuum has been reached. A vacuum of less than 1 or 2 mm. of mercury is necessary for satisfactory results, and with a good set-up with a Hyvac pump a pressure of 30 to 50 microns is obtained; this is desirable, since, in general, the better the vacuum, the more satisfactory are the ultimate results. About an hour after this high vacuum has been attained the stop cock is closed. With proper equipment and a good set-up the contents of the tubes should be dried at least by the following morning.

Before opening the desiccator the air should be allowed to enter very slowly. While it has been our custom to allow natural atmospheric air to enter the desiccator, it would probably be better to pass it over calcium chloride or some other non-volatile desiccant, for in this way one could be more certain of a minimal amount of water vapor coming in contact with the dried culture.

Sealing: With very delicate material, as for instance some of the viruses, it is probably better to use the Flosdorf-Mudd apparatus which permits sealing with the contents of the tubes under a high vacuum. If this is not available and the desiccator method is used the material should be placed in longer tubes; then, as recommended by Elser, Thomas and Steffen (1935), the tubes can be constricted in a blow pipe flame just below the level of the cotton; they may then be connected to a vacuum pump, without removing the cotton plugs, and secondarily evacuated, after which they can be closed by fusing the constricted portion of the tubes.

With most bacteria, however, we have found a wax seal applied at atmospheric pressure more suitable on account of convenience both in closing and opening the tubes. Greater safety is attained because contamination of the cultures by an inrush of air is prevented, and there is no danger of a spread of the dried bacteria into the surrounding atmosphere. Consequently, we have employed the following technique: The tubes are removed from the

desiccator, freed of glycerol, and placed in rows in suitable racks. *Without delay* melted wax is allowed to run into the space above the cotton. The wax must be *very hot* in order to adhere well to the glass and make a perfect seal. A micro-bunsen burner is very satisfactory for melting the wax and heating the top of the tubes so that the melted wax drops directly into the space above the cotton plug. Air bubbles in the wax are removed by secondarily heating the tubes as they are rotated. Following this, a second application of wax is made. We have used successively paraffin wax, sealing wax, and finally Picein, as seals. The first has the disadvantage of not making a permanently tight seal; consequently the plug moves, especially in hot weather (see tube 6, figure 2); sealing wax has been better, but is liable to break if the tubes are placed in the refrigerator or other cold environment; and although Picein is less liable to break than sealing wax because it is less brittle, we have observed, even with this substance, that rapid chilling may induce thin cracks between the Picein seal and the wall of the tube. For this reason we have kept the dried cultures in file boxes at room temperature rather than in the cold, and have found them to remain unaltered in their virulence, serological and other biological characteristics, for many years (probably indefinitely if the seals remain intact).[6]

The tubes are conveniently opened as follows: A piece of iron wire (we use a straightened-out paper clip) is heated red hot and pushed through the wax, then allowed to cool. When the wax is cool, the upper end of the tube is heated gently in the pilot flame of a bunsen burner so that the outer part of the wax plug is melted while the core remains solid; the plug is then easily withdrawn by means of the wire previously inserted. The cultures are recovered by placing suitable fluid media directly in the tube. In those instances where the organisms grow best on solid medium the dried material may be fished out and inoculated on suitable media.

[6] It is probable that a cement, such as Dipar Liquid Sealing Cement, recommended by Flosdorf and Mudd (1935) painted over the surface of the wax plugs and the top of the glass tubes, would make a more permanent seal resistant to the cracking that comes from cooling the tubes. Such a final sealing is especially to be recommended when the tubes are to be shipped long distances or exposed to winter temperatures in which they may be overcooled.

Properly dried material has a characteristic appearance which varies according to the substance used for suspending the bacteria. With broth the dried material will have a white or light yellow

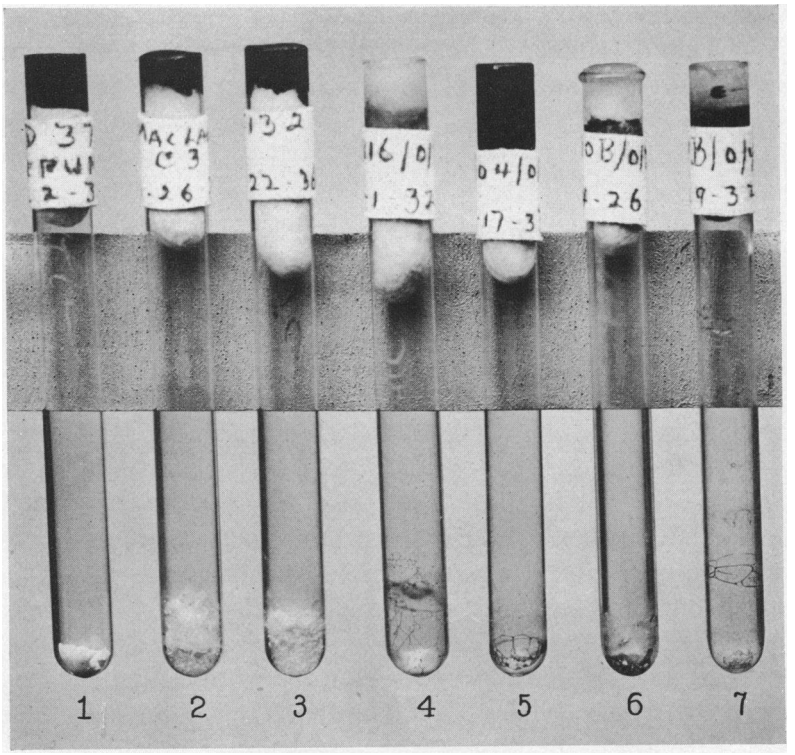

FIG. 2. APPEARANCE OF FROZEN AND DRIED CULTURES UNDER VARIOUS CONDITIONS

1, properly dried culture originally suspended in serum. 2 and 3, properly dried cultures originally suspended in broth. 4, culture which was properly dried but which is becoming gummy due to breaking of paraffin wax seal. 5 and 6, cultures which have become gummy due to breaking of seals. Note how the seal in tube 6 has been pushed out during the hot weather. 7, culture probably melted before being completely dried. Note films of dried material high up on the wall of the tube. Cultures in tubes 5, 6 and 7, not viable; and that in 4 of doubtful viability.

color and a loose frothy consistency somewhat like dried shaving lather (tubes 2 and 3, fig. 2). If serum was used as a suspending medium, the material will remain in the shape of a solid compact

cap and have a firmer appearance (tube 1, fig. 2). If the culture has melted before being completely dried, it will appear to have boiled, with a thin film high up on the side of the container and a yellow, thick, gummy film adhering tightly to the bottom of the tube (tube 7, fig. 2). Moreover, if properly dried material takes up much moisture from the air it will assume the same gummy aspect (tubes 4, 5 and 6, fig. 2). Such cultures will usually not be viable.

ORIGINAL METHOD—METHOD A

Where CO_2 ice is not available, the original method (Swift, 1921) which we employed for many years, can be easily carried out. The tubes of concentrated culture are immersed in a salt-ice mixture until the contents are well frozen. They are then transferred to the desiccator to be dried, with the precaution of carefully wiping all salt water from the outside of the tubes before they are immersed in the glycerol. The desiccator is also previously immersed in a salt ice mixture which chills the glycerol to about $-6°C$. After the tubes are all in place, a dish of P_2O_5 is placed above them, the desiccator is sealed with the paraffin-vaseline mixture and the air evacuated with a Hyvac pump. A good vacuum is essential for this method also. The desiccator is kept cold by immersion in a salt-ice mixture, or in a brine bath connected with a refrigerating system. Suitable modifications in the ordinary electrical refrigerators found in many laboratories, might also be applicable to the maintenance of sub-zero C. temperatures. Again, it should be emphasized that the cultures must be kept frozen until they are completely dried, which is usually accomplished overnight. Melting is more liable to take place in Method A than in Method B; and, as has been mentioned, it is possible that freezing at the very low temperature of $-76°C$., as is done in Method B, has advantages.

After the cultures are dry the remainder of the procedure is exactly the same as that described for Method B. Adhesive plaster marked with water-proof India ink makes a satisfactory label; for it is to be noted that good permanent labels are especially desirable on cultures which are to be stored for an indefinite period.

SUMMARY

1. Bacteria maintain their original cultural, immunological and biochemical characteristics and their virulence for many years at room temperature when they are completely dried while in a frozen state, provided the cultures so dried are well sealed to keep out water vapor.

2. Two methods are described for freezing and drying material. Both require the maintenance of a high vacuum until the cultures are dry and both employ P_2O_5 as a desiccant. In Method A the cooling system is a salt-ice mixture which is used both for freezing the cultures and for reducing the glycerol in the desiccator to a sub-zero temperature, and some special provision must be made for keeping the cultures frozen until desiccation is complete. In Method B the cultures are frozen in the desiccator by immersion in glycerol to which solid CO_2 is added; and suitably low temperatures are maintained by placing the desiccator in a cork insulated box, containing a small amount of CO_2 ice. Either Picein or sealing wax make satisfactory seals provided the tubes are never subjected to ice-box temperatures.

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